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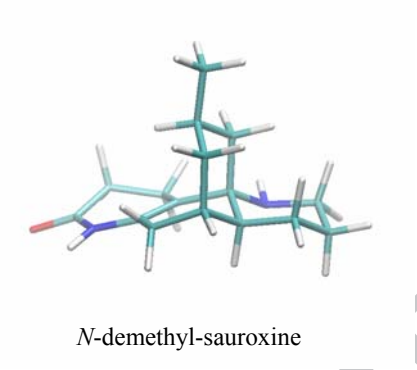
GRAPHICAL ABSTRACT



N-demethyl-sauroxine

N-demethyl-sauroxine, a novel Lycodine Group alkaloid from *Huperzia saururus*

Mariana G. Vallejo, Maria Gabriela Ortega, José L. Cabrera and Alicia Mariel Agnese*.

Farmacognosia, Departamento de Farmacia, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba- Instituto Multidisciplinario de Biología Vegetal, (IMBIV-CONICET). Edificio de Ciencias 2, Medina Allende y Haya de la Torre, Ciudad Universitaria, Córdoba X5000HUA, Argentina.

The present study describes the isolation and identification of *N*-demethyl-sauroxine, a novel Lycopodium alkaloid obtained from *Huperzia saururus* (Lam.) Trevis. (Lycopodiaceae). Its structure and relative stereochemistry were elucidated on the basis of its spectral data and chemical correlations. Additionally, acetylcholinesterase inhibitory activity was evaluated (IC_{50} = 209.6 ± 1.1 μM). The structure of the already identified alkaloid sauroxine was also re-validated through two dimensional NMR data.

Keywords: Huperzia saururus; *N*-demethyl-sauroxine; acetylcholinesterase inhibition; sauroxine NMR two dimensional data.

*Corresponding author. Tel.: 54-351-5353865; fax: 54-351-5353865. *e-mail address*: magnese@fcq.unc.edu.ar. (Alicia Mariel Agnese).

Huperzia saururus (Lam.) Trevis. ("cola de quirquincho", Lycopodiaceae) is a native Argentine species, used in the ethnomedicine as aphrodisiac[1] and memory improver.[2] As a consequence of decoctions consuming, cases of intoxication were detected, with manifestations such as nauseas, vomits, abortion, even comma as it is explained by the Health Ministry in Argentina.[1,3]

Regarding chemical studies, we have identified *Lycopodium* alkaloids as the main compounds being sauroine and sauroxine (**1**) the majority alkaloids.[4,5] According to the hypothetic cholinergic stimulation implied by the adverse effects, biological assays developed with the alkaloid extract (AE) showed an important inhibitory action on the acetylcholinesterase enzyme (AChE).[4] Also it was demonstrated that AE exerts a facilitation of both, the long term potentiaton (LTP) induction on hippocampal slices[6] and the memory retention according to Step-down Test results.[7] Besides, sauroine has similar properties in relation to the memory phenomenon, but it has not an inhibitory effect on AChE.[5,8]

In this paper, the isolation and structure elucidation of a novel *Lycopodium* alkaloid (**2**) is described. The obtaining of the new compound through a semi-synthesis strategy was also proposed, employing sauroxine, the second major alkaloid in *H. saururus,* as a substrate.

Additionally, due to the limited existing data of sauroxine,[9,10] its structure was re-validated.

PREFERRED POSITION FOR CHEMICAL STRUCTURES

Aerial parts of *H. saururus* dried and ground (2.45 kg) were alkalinized with 0.1 M NaOH (pH 12) and extracted by soxhlet with $ClCH_3$ as solvent. 77 g of extract were obtained. This was solubilized with pH 2 HCl solution, then alkalinized until pH 12 with 0.1 N NaOH and partitioned with $ClCH_3$ by using a liquid-liquid extractor. The organic phase, with a yield of 3.95 g, was purified by Sephadex LH-20 using $ClCH_3$/EtOH (1:1) as mobile phase. All the fractions that were positive to the Dragendorff`s reagent were combined (2.21 g). This fraction was re-

submitted to Sephadex LH_{20}, but acetone was used as mobile phase. Fraction 3 was purified by CC using Sephadex G-10 with H_2O/EtOH (95:5). By posterior purification of the third fraction by TLC using ciclohexane/$ClCH_3$/diethylamine (5:4:1) as mobile phase, **1** (9.7 mg, 0.00039%) was obtained; fraction 4 was purified in the same way but using ciclohexane/diethylamine (1:1) as mobile phase. Three bands were obtained, and the first one was purified by TLC with Cl_2CH_2/MeOH (4:1) leading to *N*-demethyl-sauroxine (**2,** 1.9 mg, 0.0000775%). It is noteworthy that this alkaloid was also obtained by using other extraction methods, and other extractant such as H_20, both at acid and neutral pH (data not shown), thus dismissing the possibility that **2** could be an artefact.

Semi-synthesis of **2** was carried out by means of an adaptation of the Polonovsky reaction.[11] **1** (9.1 mg) was solubilized in MeOH (200 µL) and maintained at 0ºC. Then, H_2O_2 (20 µL) was added drop by drop. Reaction was left to develop at 25ºC, through 6 h. Later, little amounts of MnO_2 were added to quenching the H_2O_2 excess, and the necessary amount of MnO_2 was detected with KI-starch paper. This suspension was filtered by Celite® and taken to dryness, then solubilized in MeOH (200 µL) and, HCl 6 M was added until pH 2. Solution was taken to dryness again. Thus, sauroxine *N*-oxide hydrochloride was obtained (10.7 mg). This was dissolved in MeOH (200 µL) and $FeSO_4$•$7H_2O$ (2 equiv) was added. This second step of reaction was developed as well at 25ºC through 30 min. The solution was taken to dryness and solubilized with EDTA 0.1 M at pH 10 and then it was partitioned with Cl_3CH ten times. Organic fractions were reunited and $MgSO_4$ (1.2 mg) was added. After filtration, it was taken to dryness. Thus, a mixture of **1** and **2** was obtained.

Both alkaloids were immediately separated by TLC, with ciclohexane/Cl_3CH/dietilamine (5:4:1) as mobile phase. This way, 1.2 mg of **2** were obtained. Semi-synthetic and natural compounds were compared chromatographically by TLC.

Sauroxine (**1**). The structure of **1** was re-validated by the 2D NMR (^{1}H-^{1}H COSY, HMQC, HMBC, NOESY) data. The ^{1}H-^{1}H COSY and TOCSY (Figure 1) revealed the connectivities of

C-2 to C-3, C-6 to C-7 and C-8, C-9 to C-11, and C-14 to C-16. The HMBC data (Figure 1) confirmed the α-pyridone ring: H-2 showed correlations with C-1, C-4 and C-5, H-3 with C-2 and C-4, and N_{α}-H with C-4. Connection to the B ring is showed through the following correlations: H-6 with C-4 and C-5, and H-7 with C-4 and C-5. Connectivities in B ring were H-6 to C-7, C-8 and C-12, H-7 to C-12 and C-13, and H-8 to C-6, C-7 and C-12. Piperidine C ring was constituted according to the correlations between H-9 with C-11 and C-13, H-10 with C-12, H-11 with C-9 and C-10, and H-12 with C-11. Finally, D ring correlations were H-7 with C-15, H-14 with C-15, H-16 with C-8, C-14 and C-15, and H-14 with C-4. NOESY correlations (Figure 1) allowed the statement of the stereochemistry of **1**. The most important were the existing between H-12 and H-6b, and H-3a.

Around thirty *Lycopodium* alkaloids belong to the Lycodine Class, where C/D mostly exhibit the *trans* configuration[12], α-obscurine being one example.[13] Differently, **1** is an stereoisomer of α-obscurine and for this reason it is also called 12-epi-α-obscurine. In 1974, Nakashima et al., showed differences in ^{13}C NMR spectrum between the carbon signals at C-8 and C-14 for **1** and α-obscurine[10]. In the present work, we improve the analysis showing these differences through 2D NMR, supporting the unique conformation of C/D ring in **1** and **2**.

N-demethyl-sauroxine[14] (**2**). The IR absortions implied the presence of a ketone carbonyl group (1665.80 cm^{-1}), an amide and amine group at 3223 cm^{-1} and 3382 cm^{-1}, respectively. **2** showed a molecular ion at m/z 260 (12.5 %) consistent with the formula $C_{16}H_{25}N_2O$ deduced from the HRESIMS (found: 261.1973; calcd; 261.1967). The base peak at m/z 203 (100 %) indicates the loss of the bridge ring (C-8, C-14, C-15, and C-16), plus a hydrogen atom from C-12 suggesting that **2** possess a lycodine-type skeleton[15].

The ^{13}C analysis and HSQC-DEPT spectra indicated 16 carbon signals and showed the presence of only one methyl carbon, three methine carbons, eight methylene carbons, and four quaternary carbons. The carbon signals at δ_C= 170.8 and δ_C= 59.5 correspond to the carbonyl group (C-1) and the quaternary function (C-13).The signals at $\delta_{C\text{-}4}$= 110.6 and $\delta_{C\text{-}5}$= 133.6

correspond to the double bond C-C. HMBC experiment established that those signals have not correlations with any proton (Table 1). Comparing the ^{13}C NMR spectrum of **1** and **2,** the most important differences were one less carbon signal and the absence of the signal belonging to the methyl group (N_β-CH_3) of **1**. Furthermore, there is an increase of $\delta_{C\text{-}10}$= 18.6 (22.8), and $\delta_{C\text{-}12}$= 32.6 (40.1) signals, and a decrease of $\delta_{C\text{-}9}$= 47.6 (39.9) and $\delta_{C\text{-}11}$= 24.9 (22.4). All this is consistent with the effect observed in lycodine, in comparison with *N*-methyl-lycodine, where the *N*-methyl group is axially oriented as well.[10]

The ^1H-^1H COSY and TOCSY (Figure 2) revealed the connectivities of C-2 to C-3, C-6 to C-7 and C-8, C-9 to C-11, and C-14 to C-16. The HMBC data (Figure 2) led to confirm the α-pyridone ring: H-2 showed correlations with C-1, C-3 and C-4, H-3 with C-4 and C-5, and N_α-H with C-4. Connection to the B ring is showed through the following correlations: H-6 with C-4 and C-5. Connectivities in B ring were H-6 to C-7, C-8 and C-12. Connectivity in the D ring was H-8 to C-12. Finally, D ring correlations were H-14 with C-4, C-13 and C-15, H-16 with C-8, C-14 and C-15.

NOESY correlations (Figure 2) allowed the statement of the stereochemistry of **2**. The most important were those existing between H-12 and H-6b, and H3a. Together with the previous spectroscopic data, we can determine the C/D ring configuration as *cis*, and therefore, **1** and **2** are the only two Lycodine Group alkaloids having this conformation.

Semi-synthetic **2** was obtained as an amorphous solid, at a very low yield. Nevertheless, some important signals were detected in the ^1H NMR spectrum such as the methyl group in C-16 and again, the absence of a methyl group in N_β compared to **1**. In addition, several carbon signals were identified: C-16 (22.1), C-2 (30.8), C-7 (33.8) and C-12 (42.6). As in the natural product, these chemical shifts are different from those observed in **1**.

As it was previously mentioned, the purified alkaloid extract of *H. saururus* was previously evaluated in our lab in relation to its effect on the acetylcholinesterase enzyme. As the results obtained exhibited a strong inhibitory effect (IC_{50}= 0.58 μg/mL)[4], we evaluated some of the

purified alkaloids as well, searching for the one with the best results. Thus, sauroine showed no inhibitory effect[5], sauroxine had an IC_{50} = 8.9 ± 0.4 μg/mL (32.3 μM)[17], 6-hydroxylycopodine showed an IC_{50} = 78.1 ± 3,5 μg/mL (298.8 ± 13.3 μM)[17], and in the present paper we evaluated the same activity for **2**. An IC_{50} = 54.5 μg/mL (209.6 ± 1.1 μM) was obtained. As it can be seen, hitherto no compound has similar inhibitory activity to the extract, so we postulate a possible synergistic effect among the alkaloids present.

Acknowledgements

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Supplementary data

Supplementary data associated to this article can be found in the online version, at…

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14. *N*-demethyl-sauroxine{**2**, very pale yellow oil; $[\alpha]_D^{26}$ +17.46 (c 0.32, MeOH)}, IR (BrK) ν_{max} 3383, 3223, 2929, 1741 and 1666 cm^{-1}.

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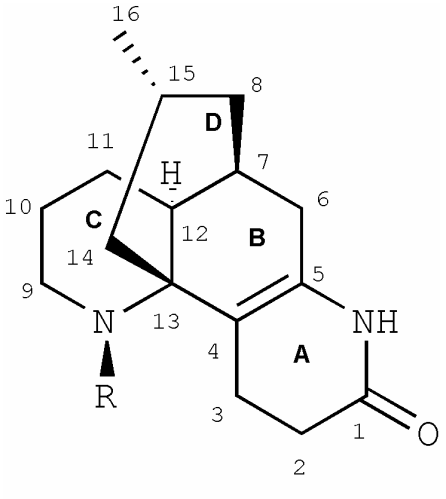
Table 1. NMR data of *N*-demethyl-sauroxine

position	δ_C [a]	δ_H [b] (multi, *J* in MHz)
1	170.8 (C)	---
2a	30.5 (CH_2)	2.42 (1H, br t, 7.7)
2b		2.68 (1H, br t, 7.7)
3a	19.7 (CH_2)	2.52-2,59 (1H, m)
3b		2.75 (1H, br t, 10.1)
4	110.6 (C)	---
5	133.5 (C)	---
6a	34.4 (CH_2)	1.84 (1H,d, 18,8)
6b		2.60 (1H, dt, 18.8; 4,3)
7	32.7 (CH)	2.06-2.11 (1H, m)
8a	35.5 (CH_2)	1.31-1.36 (1H, m)
8b		1.42 (1H, br d, 13,3)
9a	39.9 (CH_2)	3.02 (1H, t, 12.3, 3.0)
9b		3.19 (1H, d, 12.3)
10a	22.4 (CH_2)	1.50-157 (1H, m)
10b		1.82 (1H, br d, 12.2)
11	22.8 (CH_2)	1.88-2.02 (1H, m)
12	40.1 (CH)	2.03-2.10 (1H, m)
13	59.5 (C)	---
14	32.2 (CH_2)	1.65-1.73 (1H, m)
15	26.2 (CH)	1.72 (1H, m)
16	21.7 (CH_3)	0.95 (3H, d, 5.7)

[a] 100 MHz, $CDCl_3$
[b] 400 MHz, $CDCl_3$



1: R = Me
2: R = H

Figure 1.Selected 2D NMR correlations for **1**.

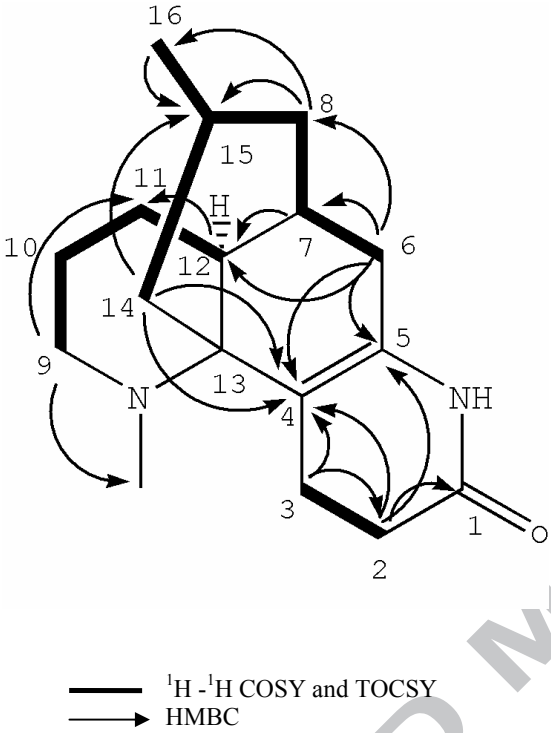
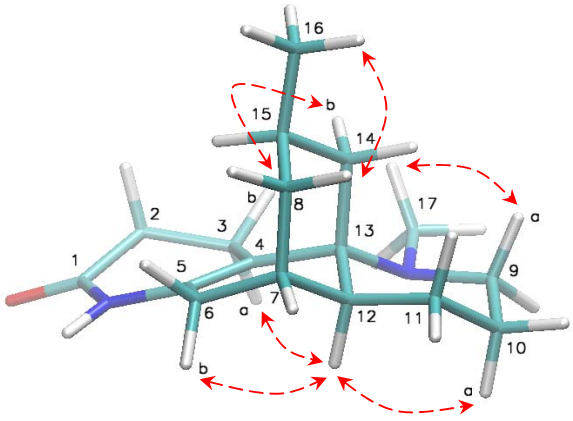
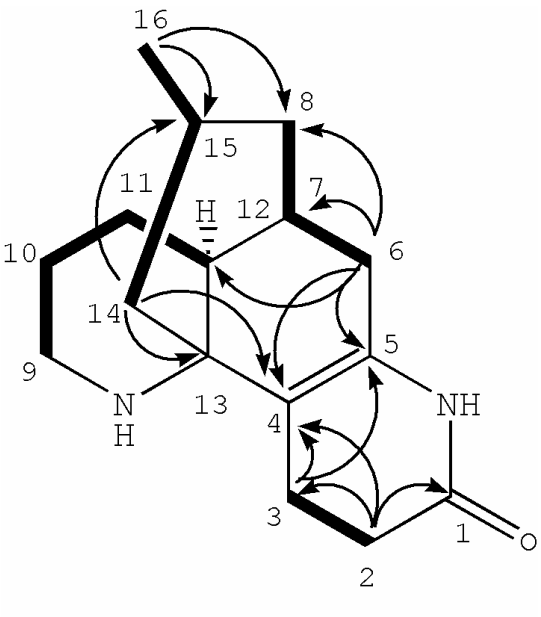


Figure 2. Selected NOESY correlations for **1**.



NOESY

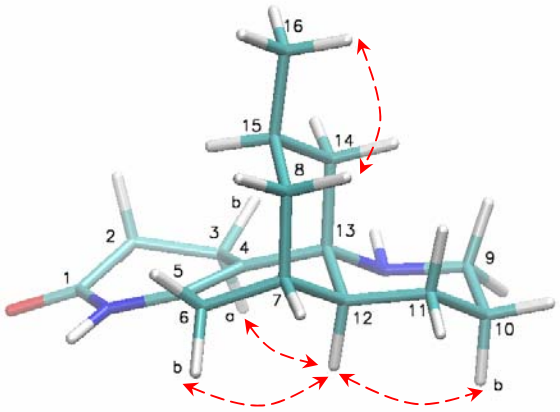
Figure 3. Selected 2D NMR correlations for **2**.



^{1}H -^{1}H COSY and TOCSY

HMBC

Figure 4. Selected NOESY correlations for **2**.



NOESY